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Posttranscriptional Regulation of the *Sesbania rostrata* Early Nodulin Gene *SrEnod2* by Cytokinin[1]

David L. Silver, Alexandre Pinaev, Rujin Chen, and Frans J. de Bruijn*

Michigan State University-Department of Energy Plant Research Laboratory (D.L.S., A.P., R.C., F.J.d.B.), Genetics Program (D.L.S., F.J.d.B.), Department of Biochemistry (R.C.), and Department of Microbiology (F.J.d.B.), Michigan State University, East Lansing, Michigan 48824

The mRNA from the *Sesbania rostrata* early nodulin gene *SrEnod2* accumulates in response to cytokinin application. Nuclear run-on assays using isolated root nuclei have shown that this accumulation occurs posttranscriptionally, and northern blot analysis of nuclear and total RNA levels revealed that it occurs primarily in the cytoplasm and not in the nucleus. After cytokinin enhancement of *SrEnod2* mRNA accumulation and the subsequent removal of cytokinin, the levels of *SrEnod2* mRNA did not return to basal levels, but oscillated over a 36-h time course. Application of the translational inhibitor cycloheximide was found to inhibit the enhancement of *SrEnod2* mRNA accumulation by cytokinin and to cause its rapid decay. Okadaic acid and staurosporine, inhibitors of protein phosphatases and kinases, respectively, also inhibited cytokinin enhancement of *SrEnod2* mRNA accumulation. In addition, okadaic acid was found to cause a decrease in *SrEnod2* mRNA levels. These results provide evidence for a posttranscriptional mechanism of cytokinin enhancement of *SrEnod2* mRNA accumulation, which appears to require concurrent protein synthesis, to involve protein phosphatases and kinases, and to occur primarily in the cytoplasm of the plant cell.

The plant hormone cytokinin comprises a group of plant growth substances that are derived from adenine. It has been shown that cytokinins induce cell division and organogenesis in cell cultures (Skoog and Miller, 1957), and also affect other physiological and developmental plant processes (Evans, 1984; Brzobohaty et al., 1994; Davies, 1995). Cytokinin and auxin have been shown to play a central role in photomorphogenesis and elongation growth (Hobbie et al., 1994). The available information on auxin signal transduction has been accumulating rapidly with the identification of auxin-induced mRNAs and auxin-binding proteins, the cloning of putative auxin receptors and auxin-responsive DNA elements, as well as the characterization of mutants in auxin responses (for review, see Hobbie et al., 1994) and the cloning of a gene responsible for one of the auxin-resistant mutant phenotypes (Leyser et al., 1993).

Only a limited number of cytokinin response mutants have been isolated (Moffatt et al., 1991; Chaudhury et al., 1993; Deikman and Ulrich., 1995), and the genes corresponding to these mutant loci have yet to be identified. Putative cytokinin-binding proteins have been purified (Brzobohaty et al., 1994), but the demonstration of their biological activity is still lacking. Overall, little is known about the molecular mechanisms of cytokinin signal transduction.

At the molecular level, cytokinin has been shown to modulate enzyme activities (Treharne et al., 1970; Chatfield and Armstrong, 1986) and transcript levels of a variety of genes. mRNA accumulation is enhanced by cytokinins, including those encoded by a wheat protein kinase gene (Sano and Youssefian, 1994), the gene for the small subunit of Rubisco (*RbcS*) (Flores and Tobin, 1988), the chlorophyll *a/b* binding protein gene (*Cab*) (Flores and Tobin, 1988), defense-related genes (Memelink et al., 1987), the PEP carboxylase gene (*PepC*) (Suzuki et al., 1994), nitrate reductase genes (Lips and Roth-Bejerano, 1969; Dilworth and Kende, 1974; Lu et al., 1990), the pollen allergen gene (*Cim1*) (Crowell, 1994), the multiple stimulus response gene cDNA (pLS216) (Dominov et al., 1992), genes of the anthocyanin biosynthetic pathway (Deikman and Hammer, 1995), cyclin D homologs (Soni et al., 1995), and genes for a number of unidentified cDNAs (Crowell et al., 1990). It was shown by nuclear run-on assays that the expression of genes encoding PEP carboxylase (Suzuki et al., 1994), nitrate reductase (Lu et al., 1990), chalcone synthase, and dihydrofolate reductase (Deikman and Hammer, 1995) are enhanced primarily at the transcriptional level by cytokinin. On the contrary, nuclear run-on assays show that some genes are regulated by cytokinin primarily at the posttranscriptional level. These genes include those encoding the chlorophyll *a/b* binding protein, the small subunit of Rubisco (Flores and Tobin, 1988), chalcone isomerase, and Phe ammonia-lyase 1 (Deikman and Hammer, 1995).

The genes involved in the cytokinin-induced accumulation of mRNA are diverse, as are the mechanisms of cytokinin induction. Cytokinin appears to enhance the transcription of genes or enhance mRNA accumulation posttranscriptionally. It is important to note that in all of the examples reported thus far, cytokinin enhancement of gene expression is never exclusively the result of cytokinin action, but is generally co-mediated by other environmen-

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* Corresponding author; e-mail debruijn@msu.edu; fax 1–517–353–9168.

Abbreviation: BAP, benzylaminopurine.

tal factors such as light, nitrogen, carbon, or other plant hormones. This has complicated the analysis of the molecular basis of cytokinin action. One exception is the *Sesbania rostrata* early nodulin gene *SrEnod2*. The *SrEnod2* gene encodes a Pro-rich protein expressed in a cell-specific manner in the nodules of legumes (van de Wiel et al., 1990). This cell layer, which surrounds the cells infected with nitrogen-fixing bacteria, is called the nodule parenchyma. It has been previously shown by Dehio and de Bruijn (1992) that the *SrEnod2* mRNA accumulates in *S. rostrata* roots in the absence of rhizobia in a time- and concentration-dependent manner in response to cytokinin treatment. The root cell type in which the *SrEnod2* gene is expressed has not been determined. This accumulation of *SrEnod2* mRNA in unnodulated roots occurs primarily in the primary root, and to a lesser extent in fully developed lateral roots, which correlates well with the observed GUS expression pattern in transgenic *Lotus japonicus* plants harboring *SrEnod2-GUS* fusions (D.L. Silver and F.J. de Bruijn, unpublished data). *SrEnod2* is a good gene with which to study cytokinin signal transduction, since its mRNA accumulation is stimulated solely by cytokinin (Dehio and de Bruijn, 1992; Hirsch and Fang, 1994). We sought to determine the most important parameters affecting this process. We demonstrate that *SrEnod2* mRNA accumulation is posttranscriptionally enhanced by cytokinin, and that this mechanism requires ongoing protein synthesis, involves protein phosphatases and kinases, and occurs primarily in the cytoplasm.

MATERIALS AND METHODS

Sesbania rostrata seeds were germinated and seedlings grown in soil composed of Metro Mix (Hummert International, Earth City, MO) and sand (2:1) at 30°C, with an 18-h light, 28°C/6-h dark, 22°C regime for 2 weeks in growth chambers with 75% RH. For all chemical treatments, plants were washed free of soil and incubated in a 1:2 dilution of minimal organic medium (Murashige- Skoog, GIBCO-BRL), along with the appropriate chemicals, under normal growth conditions. 6-BAP (Sigma), cycloheximide (Sigma), okadaic acid (GIBCO-BRL), and staurosporine (Sigma) were used at concentrations of 10, 140, 0.5, and 10 μM, respectively. Following chemical treatments, root tissues were excised, frozen in liquid nitrogen, and stored at −80°C.

Isolation of Nuclei and Analysis of Nuclear Run-On Transcripts

Frozen roots were ground in liquid nitrogen to a fine powder. The powder was resuspended in nuclei isolation buffer (20 mM Mes, pH 6.5; 2.5% Ficoll 400; 2.5% Dextran 40,000 [Sigma]; 50 mM KCl; 0.44 M Suc; 0.1% thiodiglycol; 0.5 mM spermidine; 0.1 mM spermine; 0.5 mM EDTA; 0.5% Triton X-100 [Sigma]; and 5 μg/mL aprotinin, leupeptin, and leupeptin). The resuspended material was passed through four layers of cheesecloth, two layers of Miracloth (Calbiochem), and one layer of 100-μm mesh and spun at 2,500 rpm for 15 min (HB4 rotor, Sorvall). The pellet was resuspended in nuclei isolation buffer, passed through a 20-μm mesh, and spun at 2,000 rpm for 15 min. The pellet was resuspended in nuclei isolation buffer. Nuclei were counted using the DNA stain 4′,6-diamidino-2-phenylindole dihydrochloride, and aliquots of 2×10^6 nuclei were frozen at −80°C. Nuclear run-on assays were performed as described by DeRocher and Bohnert (1993). Slot-blot filters containing 5 μg of linearized plasmid DNA containing the *SrEnod2* coding region (Dehio and de Bruijn, 1992), pUC19 (New England BioLabs), and the *βATPase* gene (Boutry and Chua, 1985) on nitrocellulose membrane (Bio-Rad) were used to hybridize with equal counts of transcripts (5×10^7 cpm). Filters were washed at 65°C in 2.0× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% SDS for 20 min; 0.5× SSC, 0.1% SDS for 20 min; and 0.1× SSC, 0.1% SDS for 30 min. The signals were quantified using PhosphorImager analysis (Model 400B, Molecular Dynamics, Sunnyvale, CA).

Isolation of Nuclei for RNA Extraction

Nuclei were isolated as described by Peters and Silverthorne (1995) with the following modification: β-mercaptoethanol was replaced by 10 mM of the RNase inhibitor ribonucleoside-vanadyl complex (GIBCO-BRL). Nuclei were resuspended in RNA extraction buffer, and RNA was isolated as described by Verwoerd et al. (1989).

Northern Blot Analysis

Ten micrograms of RNA was electrophoresed in 1.2% (w/v) agarose gels in Mops buffer (20 mM Mops, 1.0 mM EDTA, 5.0 mM sodium acetate, pH 7.0) containing 5.4% (v/v) formaldehyde. Gels were blotted onto a 0.22 μM nitrocellulose membrane (NitroPlus, Micron Separations, Westborough, MA). Membranes were probed with a [^{32}P]dATP-labeled DNA fragment containing the *SrEnod2* coding region generated with random priming (Boehringer Mannheim). Filters were reprobed with an 18S rRNA DNA probe as a loading control. The use of the *βATPase* gene (Boutry and Chua, 1985) and the soybean *actin* gene, pSAC3 (Shah, 1982), as further controls are indicated in the "Results". All filters were washed at 65°C in 2.0× SSC, 0.1% SDS for 20 min; 0.5× SSC, 0.1% SDS for 20 min; and 0.1× SSC, 0.1% SDS for 15 min. The signals were quantified using PhosphorImager analysis.

RESULTS

Transcription of the *SrEnod2* Gene and Accumulation of Its mRNA in Response to Cytokinin

Run-on transcription assays were performed using isolated nuclei to determine whether *SrEnod2* mRNA is regulated at the transcriptional and/or posttranscriptional level by cytokinin. Two-week-old *S. rostrata* seedlings were treated with the cytokinin BAP for time periods of 0 (no BAP), 2, 4, and 6 h, after which nuclei and RNA were isolated from root tissues. A northern blot analysis of the RNA samples is shown in Figure 1A. *SrEnod2* mRNA accumulated to levels approximately 4-fold higher than those of the control over the time course of BAP treatment, as

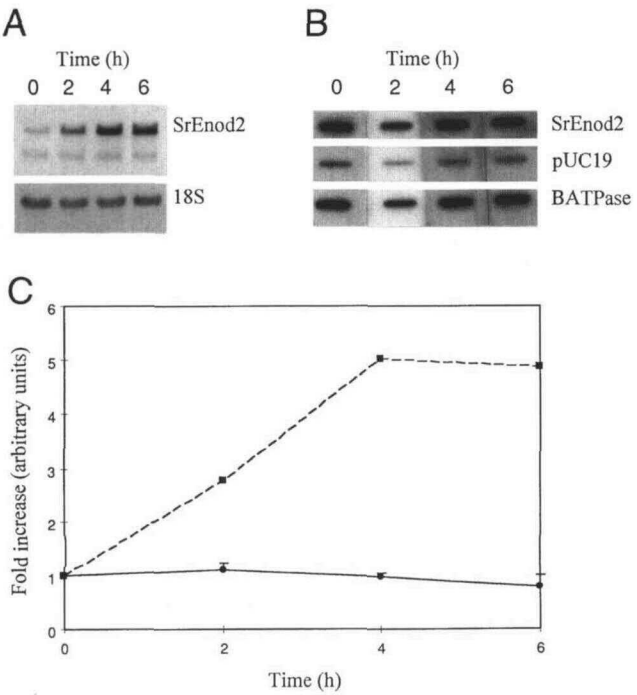

Figure 1. Comparison of nuclear run-on transcription with mRNA accumulation. A, Northern blot analysis of *SrEnod2* mRNA accumulation enhanced by cytokinin. Two-week-old *S. rostrata* seedlings were incubated in the presence of 10 μM BAP for time periods of 0 (untreated), 2, 4, and 6 h, and the total RNA was then extracted from the roots. B, Radiolabeled run-on transcripts from root nuclei isolated from the same seedlings treated in A were hybridized with immobilized *SrEnod2*, pUC19 as background plasmid control, and *βATPase* probes on slot blots. C, Signals from northern blot and run-on transcription assays were quantified using phosphor imager analysis and plotted as the increase (-fold) in signal as standardized to *βATPase* mRNA levels. The SD of two independent nuclear run-on transcription assays is shown. ●, Run-on transcription; ■, mRNA accumulation.

shown in Figure 1A. A second RNA smaller than *SrEnod2* was frequently observed on northern blots (Fig. 1A), the origin of which is unclear. This second RNA probably does not represent a second *SrEnod2* gene, since Southern blot analysis indicates that *SrEnod2* exists as a single-copy gene in *S. rostrata* (Dehio, 1989), and the northern blots were washed at high stringency. More likely, this smaller RNA may be a processing product derived from the *SrEnod2* mRNA. Radiolabeled transcripts from the nuclei were hybridized with an immobilized *SrEnod2* DNA probe on slot blots. No change in transcription of the *SrEnod2* gene was observed over the time course of BAP treatment (Fig. 1B), as indicated by the *SrEnod2* signal relative to the *βATPase* signal using phosphor imager analysis. A comparison of the northern blot and nuclear run-on data (Fig. 1C) showed that although *SrEnod2* mRNA accumulates over time, no detectable change in transcription was evident. These data suggest an involvement of posttranscriptional processes in *SrEnod2* mRNA accumulation in response to cytokinin. To examine the possibility that *SrEnod2* mRNA stability was altered in the presence of cytokinin, we examined *SrEnod2* mRNA half-life using the cellular RNA synthesis inhibitor actinomycin-D. However, the detection of differences in *SrEnod2* mRNA stability in response to cytokinin treatment has so far not been possible, because it was found that actinomycin-D stabilizes the *SrEnod2* mRNA (D.L. Silver and F.J. de Bruijn, unpublished data), making this type of analysis impossible. The effect of mRNA stabilization due to transcription inhibitors has also been observed in the case of the *PhyA* (Seeley et al., 1992), *rbcS* (Fritz et al., 1991), *PvPrP1* (Zhang et al., 1993), and Fd mRNAs (Dickey et al., 1994).

SrEnod2 mRNA Accumulation in Response to Cytokinin Occurs Primarily in the Cytoplasm

The nuclear run-on data suggest that *SrEnod2* mRNA accumulates posttranscriptionally, but do not provide insight into the question of whether *SrEnod2* mRNA accumulation is a nuclear and/or cytoplasmic event. To better understand the cytokinin signal transduction pathway, it was important to determine if *SrEnod2* mRNA accumulated in the nucleus or in the cytoplasm. Events that occur in the nucleus after transcription include pre-mRNA processing, turnover, and transport to the cytoplasm. To investigate the fate of *SrEnod2* mRNA in the nucleus, nRNA was isolated from *S. rostrata* roots treated and untreated with BAP, and analyzed by northern blot hybridization (Fig. 2A). There was only a 1.2-fold increase in *SrEnod2* mRNA accumulation in the nucleus, compared with an approximately 4-fold increase in total cellular RNA (Fig. 2B). It is important to note here that the levels of *SrEnod2* mRNA in the nucleus constitute approximately 38% of the total *SrEnod2* RNA, indicating that most of the *SrEnod2* transcripts are located in the cytoplasm. Therefore, the accumulation of *SrEnod2* mRNA appears to occur primarily in the cytoplasm and not in the nucleus.

SrEnod2 mRNA Accumulation Appears To Be a Long-Lived Process

To better define the link between *SrEnod2* mRNA accumulation and cytokinin application, we determined the level to which *SrEnod2* mRNA would increase, and to what extent the mRNA would be present over time after the removal of cytokinin. *S. rostrata* seedlings were incubated in the presence of BAP for 4 h, then divided into two groups. One group was washed extensively in water to remove BAP and was placed in BAP-free medium. Another group was further incubated in media with BAP. A third group of *S. rostrata* seedlings was not exposed to BAP and was placed continuously in BAP-free medium. All three groups of seedlings were incubated for 36 h under these conditions. Roots were harvested at 4-h intervals, and RNA was isolated and analyzed by northern blot hybridization. Neither group of seedlings exposed to BAP exhibited *SrEnod2* mRNA decay below induced levels during the 36-h time course (Fig. 3). In addition, a rhythmic oscillation with essentially the same pattern was observed in both groups treated with BAP. However, the level of *SrEnod2*

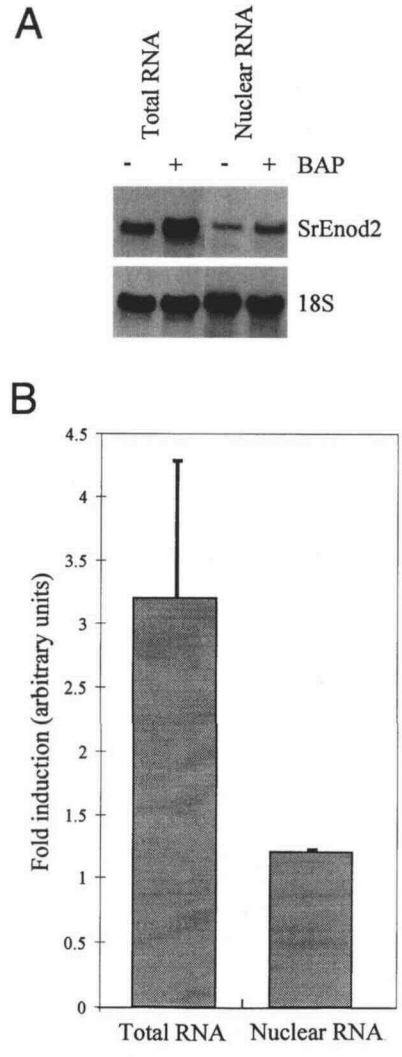

Figure 2. Abundance of *SrEnod2* mRNA derived from total RNA and nRNA. A, Representative northern blot analysis of total RNA and nRNA isolated from roots of *S. rostrata* seedlings treated with or without 10 μM BAP for 4 h. B, Quantification of *SrEnod2* mRNA accumulation in both total and nRNA. Signals from two independent experiments were quantified using phosphor imager analysis. *SrEnod2* signals were standardized to 18S rRNA signals. The SD is shown.

mRNA in samples treated with BAP for only 4 h appeared to be slightly higher than in samples treated for 36 h. The reason for this apparent difference is unclear, but may be due to signal attenuation in samples continuously exposed to cytokinin. These data may indicate that the process by which *SrEnod2* mRNA accumulates is long-lived. Whether the observed oscillation is due in part to a change in stability or synthesis of the *SrEnod2* mRNA is unknown.

Cycloheximide Inhibits *SrEnod2* mRNA Accumulation in Response to Cytokinin

It is a common observation that the level of mRNA, which is unstable, is increased by the translational inhibitor cycloheximide (Zhang et al., 1993; Dickey et al., 1994; Gil et al., 1994). There are three possible reasons for this. Cycloheximide may inhibit the accumulation of unstable components of the mRNA degradation machinery (Brewer and Ross, 1989), or translation of the specific mRNA in question may be required for its degradation (Aharon and Schneider, 1993; Byrne et al., 1993; Tanzer and Meagher, 1994). Another possibility is that cycloheximide, through a secondary effect, increases transcription (Edwards and Mahadevan, 1992). Therefore, it was of interest to determine if cytokinin could directly stimulate *SrEnod2* mRNA accumulation in the absence of translation, and/or if the *SrEnod2* mRNA exhibited properties common to unstable transcripts in the presence of cycloheximide. A typical concentration of cycloheximide used in plants is between 50 and 70 μM in cell culture (Heinhorst et al., 1985; Gil et al., 1994), and up to 300 μM (for a 2-h pretreatment) in intact plants (Mita et al., 1995). In our system 70 μM cycloheximide inhibited the accumulation of *SrEnod2* mRNA, but required slightly more than 1 h of pretreatment to be effective. A concentration of 140 μM was effective after a pretreatment of between 30 min and 1 h (data not shown). It may be that a higher concentration of cycloheximide is needed to elicit an effect in whole plants than is needed in cell culture due to the timing of uptake of the substance. Two-week-old *S. rostrata* seedlings were pretreated for 1 h with 140 μM cycloheximide. The seedlings were then treated with BAP for 4 h, after which RNA was isolated and *SrEnod2* mRNA levels were examined by northern blot hybridization. It was observed that 1 h of cycloheximide pretreatment prevented subsequent stimulation of *SrEnod2* mRNA accumulation by BAP (Fig. 4), suggesting that protein synthesis may be involved in the observed cytokinin stimulation of *SrEnod2* mRNA accumulation. However, one must be cautious when interpreting results from experiments involving cycloheximide, because of its global effect on translation.

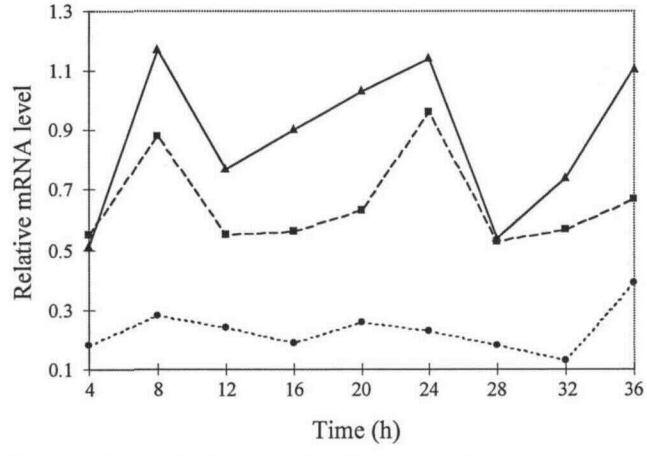

Figure 3. Fluctuation in *SrEnod2* mRNA accumulation during a 36-h period. Two-week-old *S. rostrata* seedlings were divided into three groups according to the following treatments: 36 h no BAP (●), 36 h 10 μM BAP (■), and 4 h 10 μM BAP then washed free of BAP and incubated for 32 h (▲). RNA from roots of five seedlings was isolated every 4 h for 36 h. Signals were quantified using PhosphorImager analysis. *SrEnod2* signals were standardized to *βATPase* mRNA signals.

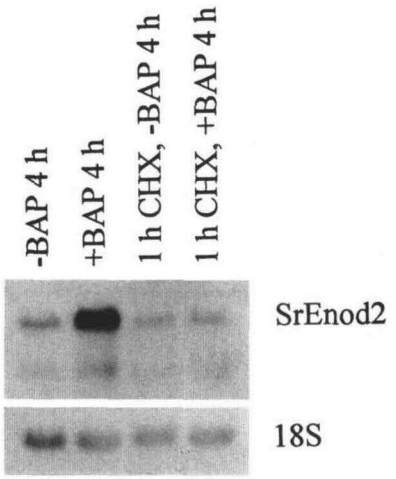

Figure 4. Effect of cycloheximide on *SrEnod2* mRNA accumulation. Two-week-old *S. rostrata* seedlings were pretreated for 1 h with 140 μM cycloheximide (CHX), and then extensively washed and further incubated for an additional 4 h with or without 10 μM BAP. Control seedlings were untreated with cycloheximide. Following the treatments, RNA from roots was isolated and analyzed by northern blot hybridization.

In addition to the inhibition of *SrEnod2* mRNA accumulation, a slight decrease in *SrEnod2* mRNA was observed in samples treated with cycloheximide with or without BAP. Because of the observed slight decrease in levels of *SrEnod2* mRNA, it was subsequently examined whether cycloheximide could cause the decay of *SrEnod2* mRNA over time after an initial BAP treatment of 4 h. Plants were treated with BAP for 4 h, after which cycloheximide was added, and RNA was harvested every hour for 4 h. Control plants were not treated with BAP, but were treated with cycloheximide. Northern blot analysis revealed that the *SrEnod2* mRNA from control plants and from plants treated with BAP decayed upon treatment with cycloheximide (Fig. 5), whereas the *SrEnod2* mRNA from plants treated with BAP but not with cycloheximide increased approximately 2-fold over this same time period of 4 h (see Fig. 3, between 4 and 8 h).

An interesting observation was made after analyzing the samples from BAP-treated plants at the 0- and 2-h time points after cycloheximide addition (Fig. 5B). Between the 0- and 2-h time points, the *SrEnod2* mRNA increased slightly, and then decayed dramatically. The apparent increase between 0 and 1 h may be explained by a lag time required for cycloheximide to be effective, but the reason for the rapid decay after the 1-h time point is not known. In addition, between 2 and 4 h after cycloheximide application, *SrEnod2* mRNA from both BAP-treated and untreated samples decayed similarly. To exclude the possibility that cycloheximide altered the levels of gene expression in general, we reprobed the northern blots with the *βATPase* gene. We found that the levels of *βATPase* mRNA were not affected by cycloheximide, suggesting that the observed effects of cycloheximide on *SrEnod2* mRNA accumulation may be specific to the *SrEnod2* gene (data not shown). Although direct evidence for an inhibition of cellular translation and/or translation of *SrEnod2* mRNA by cycloheximide has not been demonstrated under the conditions used in these experiments, these data may suggest that ongoing protein synthesis is required for the continued accumulation of both basal and cytokinin-stimulated levels of *SrEnod2* mRNA.

Both Cellular Protein Phosphatases and Protein Kinases May Be Required for the Accumulation of *SrEnod2* mRNA by Cytokinin

Since cycloheximide was found to inhibit the enhancement of *SrEnod2* mRNA accumulation by cytokinin, we postulated that cytokinin may act far upstream of the actual event that causes the accumulation of *SrEnod2* mRNA. It has been shown that cytokinins are involved in protein phosphorylation cascades (Sano and Youssefian, 1994). We sought to determine if protein phosphatases and protein kinases, the mediators of signal transduction pathways (Bowler and Chua, 1994), may be involved in this mecha-

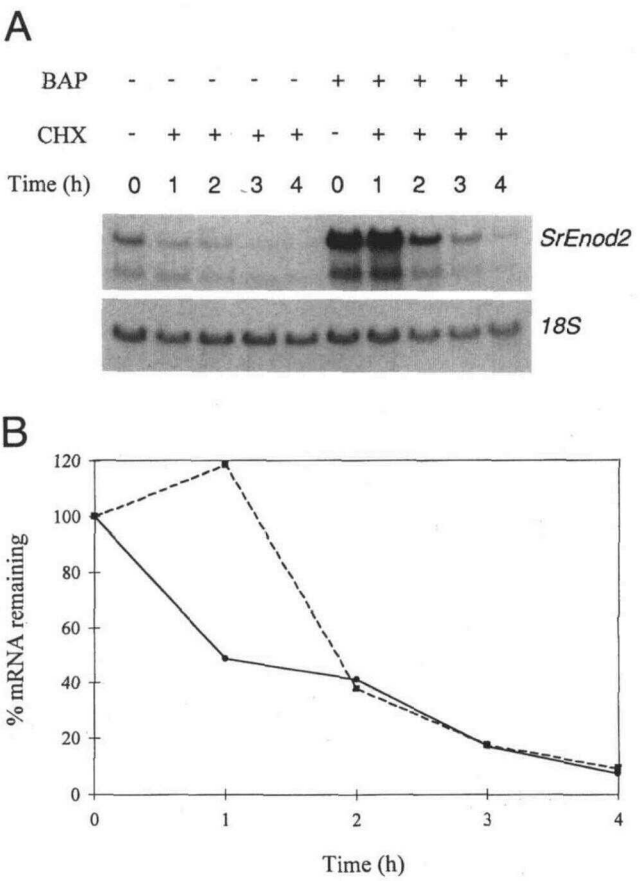

Figure 5. Decay of *SrEnod2* mRNA in the presence of cycloheximide. A, Two-week-old *S. rostrata* seedlings were initially treated with (+) or without (−) 10 μM BAP for 4 h, after which 140 μM cycloheximide (CHX) was added, and RNA from roots of five seedlings was isolated every hour for 4 h and analyzed by northern blot hybridization. B, Signals were quantified using PhosphorImager analysis. A value of 100% on the ordinate represents the maximum mRNA levels detected, as standardized to 18S rRNA signals. ■, With BAP; ●, without BAP.

nism, as has been documented for ethylene signal transduction pathways (Raz and Fluhr, 1993). To test this hypothesis, the pharmacological agents okadaic acid and staurosporine were used. Okadaic acid is an inhibitor of the protein phosphatases PP1 and PP2B at submicromolar concentrations and of PP2A at higher concentrations (Hunter, 1995). Okadaic acid and staurosporine, a broad-spectrum protein kinase inhibitor (Tamaoki, 1991), were applied at concentrations previously used for inhibitor studies in plants (Dominov et al., 1992; Raz and Fluhr, 1993; Crowell, 1994) to 2-week-old *S. rostrata* seedlings in the presence or absence of BAP for 4 h. Although okadaic acid and staurosporine are useful tools for studying signal transduction, it should be noted that the nature of their in vivo action is not known, so results obtained using these inhibitors should be interpreted with caution. Okadaic acid was found to abolish *SrEnod2* mRNA accumulation in response to BAP and to cause a dramatic decrease in the levels of *SrEnod2* mRNA in both BAP-treated and untreated *S. rostrata* seedlings below levels observed in seedlings not treated with BAP (basal level; Fig. 6). Staurosporine was also found to inhibit *SrEnod2* mRNA accumulation in response to BAP, but did not reduce the amount of *SrEnod2* mRNA below basal levels. Levels of both *βATPase* and *actin* mRNA were found to be unaffected by the inhibitor treatments (data not shown).

The observation that inhibitors of protein phosphatases and protein kinases also inhibit the accumulation of *SrEnod2* mRNA in the presence of cytokinin suggests the involvement of these classes of proteins in the cytokinin signal transduction pathway.

DISCUSSION

In this paper we present evidence for a posttranscriptional mechanism of enhancement of *SrEnod2* mRNA accumulation in response to cytokinin. Nuclear run-on experiments demonstrated that the *SrEnod2* transcription rate cannot account for the increase in *SrEnod2* mRNA accumulation in response to cytokinin treatment. In addition, the northern blot hybridization analysis of nuclear versus total RNA accumulation showed that cytokinin enhancement of mRNA accumulation appears to occur primarily in the cytoplasm. There is an approximately 4-fold enhancement in *SrEnod2* mRNA accumulation in total RNA and an approximately 1.2-fold enhancement in nRNA populations, which may be due to a small increase in transcription and/or altered mRNA stability in the nucleus. It has been documented that mRNA stability in the nucleus plays an important role in gene expression (Belgrader and Maquat, 1994; Peters and Silverthorne, 1995). Of course, it also cannot be ruled out that the observed small enhancement in nuclear *SrEnod2* mRNA accumulation is due to contamination from cytoplasmic RNA. Nevertheless, these data correlate well with the nuclear run-on data, suggesting the involvement of mRNA stability changes in response to cytokinin treatment in the cytoplasm (Jackson, 1993; Sullivan and Green, 1993; Beelman and Parker, 1995).

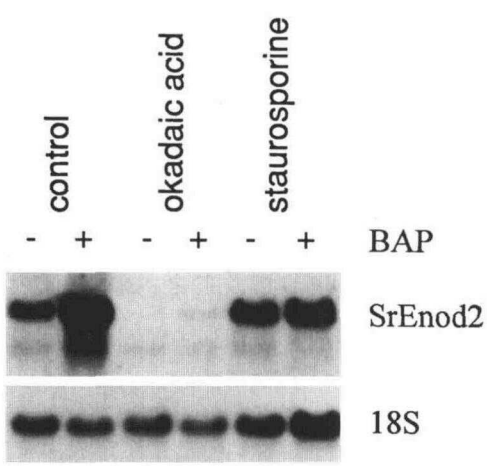

Figure 6. Effect of okadaic acid and staurosporine on *SrEnod2* mRNA accumulation. Two-week-old *S. rostrata* seedlings were preincubated for 1 h with 0.5 μM okadaic acid or 10 μM staurosporine, then treated with (+) or without (−) 10 μM BAP. RNA was isolated from roots and analyzed by northern blot hybridization.

Cytokinin seems to be acting indirectly on *SrEnod2* mRNA levels, since it was found that the inhibition of translation by cycloheximide counteracted the enhancement of *SrEnod2* mRNA accumulation by cytokinin. Cytokinin enhancement of the light-dependent expression of the nitrate reductase gene has been shown by nuclear run-on assays to be partially regulated at the transcriptional level. In addition, nitrate reductase mRNA levels do not appear to be affected by cycloheximide (Lu et al., 1990). Lu et al. (1990) proposed that concurrent protein synthesis is not required for cytokinin enhancement of the gene for nitrate reductase (*NR*) mRNA accumulation. This appears to be in direct contrast to the enhancement of *SrEnod2* mRNA accumulation by cytokinin observed here, which appears to require concurrent protein synthesis and to occur primarily at the posttranscriptional level. Surprisingly, cycloheximide was found to cause the rapid decay of *SrEnod2* mRNA from plants treated with BAP and from those that were not treated. This could be the result of an inhibition of translation of a labile protein required for *SrEnod2* mRNA accumulation, or translation of the *SrEnod2* mRNA itself may be required for its stabilization.

In contrast to the observed rapid decay of *SrEnod2* mRNA caused by cycloheximide application, the *SrEnod2* mRNA levels of plants treated with BAP and then washed free of the hormone did not return to basal levels, but oscillated for 36 h in a manner similar to those of plants continuously treated with BAP for 36 h. A circadian fluctuation of mRNA accumulation enhancement was recently reported in genes of the anthocyanin biosynthesis pathway (Deikman and Hammer, 1995). In that study, cytokinin treatment caused a dramatic dampening in the diurnal fluctuations of mRNA accumulation compared with plants grown without cytokinin, which suggests that cytokinin acts independently of the mechanism responsible for the circadian rhythm. This is in contrast to our observation that an increase in amplitude of *SrEnod2* mRNA occurs in the presence of cytokinin. It remains to be determined whether

this oscillation in *SrEnod2* mRNA is regulated in a circadian manner. In a study by Pilgrim et al. (1993) on nitrate reductase mRNA, expression of which is known to be induced by cytokinin (Dilworth and Kende, 1974; Lu et al., 1990), it was found that nitrate reductase mRNA accumulation also oscillates in a circadian fashion. These oscillations in mRNA accumulation were shown by nuclear run-on assays to occur at a posttranscriptional level; therefore, the involvement of posttranscriptional mechanisms for the oscillation in *SrEnod2* mRNA accumulation may also be plausible.

Okadaic acid and staurosporine were found to have different effects on *SrEnod2* mRNA accumulation. Okadaic acid inhibited cytokinin enhancement of *SrEnod2* mRNA accumulation, causing a dramatic decrease in its accumulation. We postulated that if okadaic acid was able to inhibit cytokinin enhancement of *SrEnod2* mRNA accumulation, then an inhibitor of protein kinases would probably cause an enhancement of *SrEnod2* mRNA accumulation in the absence of cytokinin. This was found not to be the case, since staurosporine inhibited *SrEnod2* mRNA accumulation in response to cytokinin, but did not cause a reduction in basal levels, as okadaic acid did. These data suggest the involvement of both dephosphorylation and phosphorylation processes, perhaps acting on different proteins of the cytokinin signal transduction pathway. The observation that okadaic acid and cycloheximide cause a dramatic decrease in *SrEnod2* mRNA accumulation in samples treated or untreated with BAP may indicate that both inhibitors act on the same protein(s). In addition, these data suggest that both basal and cytokinin-enhanced levels of *SrEnod2* mRNA may be due to the same mechanism of mRNA accumulation. It may be that basal levels of *SrEnod2* mRNA are caused by a small flux through the signal transduction pathway. The idea of a "flux," as described by Bowler and Chua (1994), relates to basal level activity of signaling intermediates, for example, protein kinases or phosphatases. In the case of *SrEnod2*, endogenous cytokinin levels could be responsible for maintaining a basal level of *SrEnod2* mRNA, as observed by northern blot analysis, and this increase in cytokinin levels above a threshold could cause an increase in *SrEnod2* mRNA accumulation.

Crowell (1994) showed that for cytokinin-enhanced expression of the soybean allergen gene, okadaic acid inhibits cytokinin induction, whereas for the cytokinin-enhanced accumulation of *pLS216* RNA, staurosporine inhibits enhancement of mRNA accumulation by cytokinin, and okadaic acid only slightly enhances mRNA accumulation (Dominov et al., 1992). These studies, along with the observations presented here, suggest that the phosphorylation state of proteins is important for cytokinin signal transduction. However, differences may exist in the requirement for phosphorylation among different pathways for the accumulation of mRNAs enhanced by cytokinin, as is the case for the signal transduction pathways for other plant hormones (Bowler and Chua, 1994). The evidence presented in this study strongly suggests that *SrEnod2* is stabilized in the cytoplasm in response to cytokinin. However, direct proof of regulated mRNA stability of *SrEnod2* mRNA by cytokinin remains to be provided, as does the stability of any mRNA regulated by a plant hormone. The use of a posttranscriptional mechanism of regulation of the *SrEnod2* gene may provide a more rapid means of controlling gene expression than the use of transcriptional regulation (Green, 1993).

There are questions that remain to be answered. For example, what is the role of the Enod2 protein in nodulation and the significance of cytokinin regulation of *SrEnod2* gene expression? It has been postulated by van de Wiel et al. (1990) that the Enod2 protein may play a role in creating an oxygen diffusion barrier in the parenchyma cell layer necessary for protecting the oxygen-sensitive nitrogenase enzyme in the infected cells of the nodule. A direct test of this hypothesis has not yet been concluded, so the role of the Enod2 protein in nodule development and functioning remains unclear. With regard to cytokinin regulation of *SrEnod2* gene expression, previous studies aimed at understanding the involvement of cytokinin in nodule development have indirectly shown an involvement of cytokinin in nodule development. One such study by Cooper and Long (1994) demonstrated the partial rescue of a nonnodulating *Rhizobium* strain (Nod^-) by the overexpression of the *Agrobacterium tumefaciens* cytokinin biosynthesis gene (*tzs*). The Nod^- *Rhizobium* overexpressing the *tzs* gene was capable of eliciting the production of nodule-like structures on alfalfa roots, indicating, at least in part, that a localized production of cytokinin can produce a phenocopy of nodule morphogenesis.

Preliminary transgenic work has shown the requirement of the *SrEnod2* 3′ downstream region for cytokinin-regulated tissue-specific expression in uninfected roots of the legume *Lotus japonicus* (D.L. Silver, R. Chen, and F.J. de Bruijn, unpublished data), which may also point to a posttranscriptional mechanism for the regulation of *SrEnod2* gene expression by cytokinin. It now remains to be determined whether DNA elements of the *SrEnod2* gene required for cytokinin regulation are the same as those required for tissue-specific expression in the nodule. This type of analysis may shed some light on the relationship between cytokinin regulation and the tissue-specific expression pattern of the *SrEnod2* gene in nodules and in roots. In addition, we are trying to develop the use of the *SrEnod2* gene as a model system for understanding cytokinin signal transduction, and to isolate *trans*-acting factors responsible for cytokinin enhancement of plant gene expression.

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